Supporting Information

Tumor-Targeted Inhibition of Monocarboxylate Transporter 1 Improves T Cell Immunotherapy of Solid Tumors

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Experimental Section

Synthesis and characterization of AZD-UPS NP. The PEG-*b*-PDPA copolymer was synthesized following the reported procedure using the atom transfer radical polymerization method (ATRP)[1]. To formulate drug-loaded micelles, the polymer was first dissolved in tetrahydrofuran (THF) and AZD3965 (Selleck, USA) was dissolved in dimethyl sulfoxide (DMSO). UPS polymer and AZD3965 solutions were mixed together with different mass ratio (5:1 or 10:1). For sonication method, 0.5 mL mixture was added into 4 mL distilled water dropwise under sonication. For microfluidic method, the microfluidic device was set up according to the IDEX method[2] with one central flow, two side flows and one outlet with a curved channel structure (**Figure 1a**). The diameter of the flow channel is 152 μm. AZD3965 and PDPA polymer in different ratio were dissolved in tetrahydrofuran with 9.1% dimethyl sulfoxide, and introduced to the microfluidic device through the central channel at a flow rate of 5 mL/h. Water was introduced through the two side channels at 12.5 mL/h. Upon mixing in the junction, AZD3965-loaded UPS nanoparticles (AZD-UPS NPs) were generated in a single nanoprecipitation step. The THF was removed by ultrafiltration with (100 kD) membrane for several times. Then distilled water or saline was added to adjust the UPS polymer concentration to 5 mg/mL as a stock solution.

HPLC analyses of AZD3965 were performed using the Agilent 1260 Infinity II System on a Poroshell 120 EC- C18 column, purchased from Agilent Technologies (Palo Alto, CA, USA). The eluent consists of (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile. The peak area at 14.5 min was measured and AZD3965 concentration in solution was obtained from the standard curve. The AZD3965 encapsulation efficiency (EE) and loading density (LD) were then calculated according to the following equations:

$$EE\% = \frac{Weight\ of\ AZD3965\ in\ NPs}{Weight\ of\ AZD3965\ fed\ initially} \times 100\%$$

$$LD\% = \frac{Weight\ of\ AZD3965\ in\ NPs}{Weight\ of\ NPs} \times 100\%$$

After micelle formation, the nanoparticles were characterized by transmission electron microscopy (TEM, JEOL 1200 EX model) for micelle size and morphology, and dynamic light scattering (DLS, Malvern MicroV model, He-Ne laser, $\lambda= 632$ nm) for hydrodynamic diameter.

pH-dependent drug release studies. Freshly made AZD-UPS NPs were mixed with phosphate buffered saline (PBS) with different pH values (7.4 or 6.0) at 37 ℃. At different time points, 0.5 mL AZD-UPS NP solution was removed and filtered by ultracentrifugation using 100 kD molecular weight cutoff membranes to collect the free drug. Drug concentration in the filtrate was measured by HPLC and further quantified to the percentage of total amount of loaded drugs.

To evaluate drug release profiles at multiple pH values, freshly made AZD-UPS NPs were first mixed with PBS solution at pH 11. Small volumes (1 μl in increments) of 0.1 M HCl was added to adjust the pH. The pH values were measured using a Mettler Toledo pH meter with a microelectrode. At different pH, 0.5 mL AZD-UPS NP solution was removed

after 15 min of incubation and free AZD3965 was collected by ultracentrifugation and analyzed by HPLC. Percentage of drug release was quantified as a function of medium pH.

pH titration studies. AZD-UPS NPs and drug-free UPS NPs were first diluted to 2.0 mg/mL with deionized water. Sodium chloride was added to adjust the salt concentration to 150 mM. pH of solution was adjusted to 10 using 0.1 M NaOH. pH titration was performed by adding small volumes (1 µL in increments) of 1.0 M HCl solution under stirring. The pH decrease was monitored as a function of the total added volume of HCl.

Animals and cells. All animal procedures were performed with ethical compliance and approval by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. Female C57BL/6 mice (6-8 weeks) were obtained from the University of Texas Southwestern breeding core. B16F10 cells were kindly provided by Dr. Patrick Hwu at MD Anderson Cancer Center and TC-1 cells by Dr. T. C. Wu at John Hopkins University. Both cell lines were routinely tested using mycoplasma contamination kit (R&D). Cells were cultured in DMEM medium (10% fetal bovine serum, 100 U/mL penicillin G sodium and 100 µg/mL streptomycin (Pen/Strep), non-essential amino acids, and 20 µM β-mercaptoethanol (β-ME)) at 37 °C in 5% CO_2 and the normal level of O_2.

Measurement of lactate and pH in the cell culture medium. For lactate measurement, TC-1 cells were cultured in normal DMEM medium as described above with different drugs: free AZD3965 (1 uM), intact AZD-UPS NPs (1 uM effective AZD3965 concentration), or pretreatment of AZD-UPS NPs in pH 6.0 buffer for pH-activated drug release followed by addition into the cell culture medium. After 24 h incubation, lactate concentration was measured by NOVA BioProfile 4. For pH measurement, TC-1 cells were cultured in DMEM medium (D5030, Sigma) without $NaHCO_3$, and in incubator without CO_2 for 24h, followed by measurement of medium pH.

Seahorse assay. Extracellular acidification rate (ECAR) of TC-1 cells was measured under different treatment for 2 h by Seahorse XF96 Extracellular Flux Analyzer. Basal conditions without glucose (Bas), with glucose (Glu), at maximum (Max) with the addition of oligomycin (Oli), and inhibited by 2-dexoxyglucose (2-DG).

Pharmacokinetics and biodistribution analysis. Mouse plasma were collected and added into 1 mL of methanol/water mixture (80/20, v/v) at 0.5, 6 and 24h after treatment by different regimens. Tissues (heart, liver, kidney and tumor) were collected at 24h. About 100 mg of tissues were removed, and the wet weight was recorded. The tissues were homogenized in 1 mL of methanol/water mixture (80/20, *v/v*). The plasma and tissue mixture were centrifuged and the supernatant was collected respectively, dried by a SpeedVac, then dissolved in methanol and measured by HPLC. AZD3965 were dissolved in DMSO at a concentration of 1 mg/mL. The concentrations of the samples were controlled at 0.05, 0.1, 0.5, 1, 2, 4, 6, 8, and 10 μg/mL to establish the calibration curve.

Tumor inoculation and treatment. Six to eight week old mice (n = 5-7 for each group) were injected subcutaneously with TC-1 (2×10^5) or B16F10 (2×10^5) cells into the right flank. Animals were treated by 50 mg/kg AZD3965 (administered twice daily by oral gavage for a total of 7 days), or by AZD-UPS NPs (containing 2 mg/kg AZD3965, IV injected at days 7, 10 and 13). Some groups were intraperitoneally injected with 200 μg checkpoint inhibitors (anti-mPD-1, BioXcell, BE0146) for comparison or synergy evaluation. Tumor growth was subsequently measured twice a week using a digital caliper and calculated as $0.5 \times$ length $\times$ width $\times$ height by blinded investigators. Mouse weight was also recorded. Mice were sacrificed when the tumor volumes reached 1,500 mm^3.

Toxicity analysis. Tumor-bearing mice were treated with 50 mg/kg AZD3965 (administered by oral gavage for a total of 7 days) or AZD-UPS NPs (containing 2 mg/kg AZD3965, IV injected at days 7, 10 and 13). Blood of different mouse groups were collected after 4h. Then alanine transaminase activity assay (Abcam, ab105134), aspartate

aminotransferase activity assay (Abcam, ab105135), and mouse cardiac troponin-I ELISA analysis (Life Diagnostics, Inc., Cat. No. CTNI-1-HS) were performed.

Flow cytometry analysis. Tumor tissues were digested by 1 mg/mL collagenase IV (Sigma-Aldrich) and 0.2 mg/mL DNase I (Sigma-Aldrich) for 45 min at 37 °C. Cells were then stained with anti-CD16/CD32 (Biolegend, Cat#: 101301, clone: 93), Viobility 405/520 Fixable Dye (Miltenyi), CD45.2 APC-CY7 (Biolegend, Clone: 104), CD3e FITC (Biolegend, Clone: 17A2), CD8a PE-vio615 (Miltenyi, Clone: REA601), Tetramer/PE - He2Db HPV 16 E7 (RAHYNIVTF) (MBL), PD-1 PE (Biolegend, Clone: 29F.1A12), TIM-3 APC (Miltenyi, Clone: REA602), CD4 VioBlue (Miltenyi, Clone: REA604), and Foxp3 PE (Miltenyi, Clone: REA788) antibodies, according to the manufacturer's protocols. Flow data were collected on a BD™ FACS LSRFortessa SORP flow cytometer and analyzed with FlowJo (Tree Star Inc., Ashland, OR).

Statistical analysis. Based on pilot immunization and tumor treatment studies, we used group sizes of five animals/group for immunogenicity measurements and five to seven animals/ group for tumor therapy experiments. Statistical analysis was performed using Microsoft Excel and Prism 5.0 (GraphPad). Data are expressed as means ± SEM or means ± SD. Data were analyzed by Student's t-test. All t-tests were one-tailed and unpaired, and were considered statistically significant if $p < 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ unless otherwise indicated). The survival rates of the two groups were analyzed using a log-rank test and were considered statistically significant if $p < 0.05$.

Table S1. Comparison of AZD3965-loaded UPS nanoparticles by sonication versus microfluidic method.

Method	NO.	Initial Weight Ratio		pH	Encapsulation efficiency (Mean ± SD, %)	Loading density (Mean ± SD, %)	Diameter (nm ± SD)	Polydispersity
		PDPA	AZD					

	S1	5	1	7.4	0.45±0.09	0.04±0.009	86.7±1.7	0.34
Sonication method	S2	10	1	7.4	0.71±0.04	0.14±0.008	84.5±0.5	0.28
	S3	10	1	9	5.1±0.4	0.46±0.03	82.8 ±1.9	0.22
	M1	5	1	7.4	16.9±1.6	2.8±0.3	41.4±0.8	0.21
Microfluidic method	M2	10	1	7.4	21.2±3.4	1.7±0.2	39.6±0.2	0.24
	M3	10	1	9	34.2±3.3	3.0±0.3	33.3±0.2	0.15
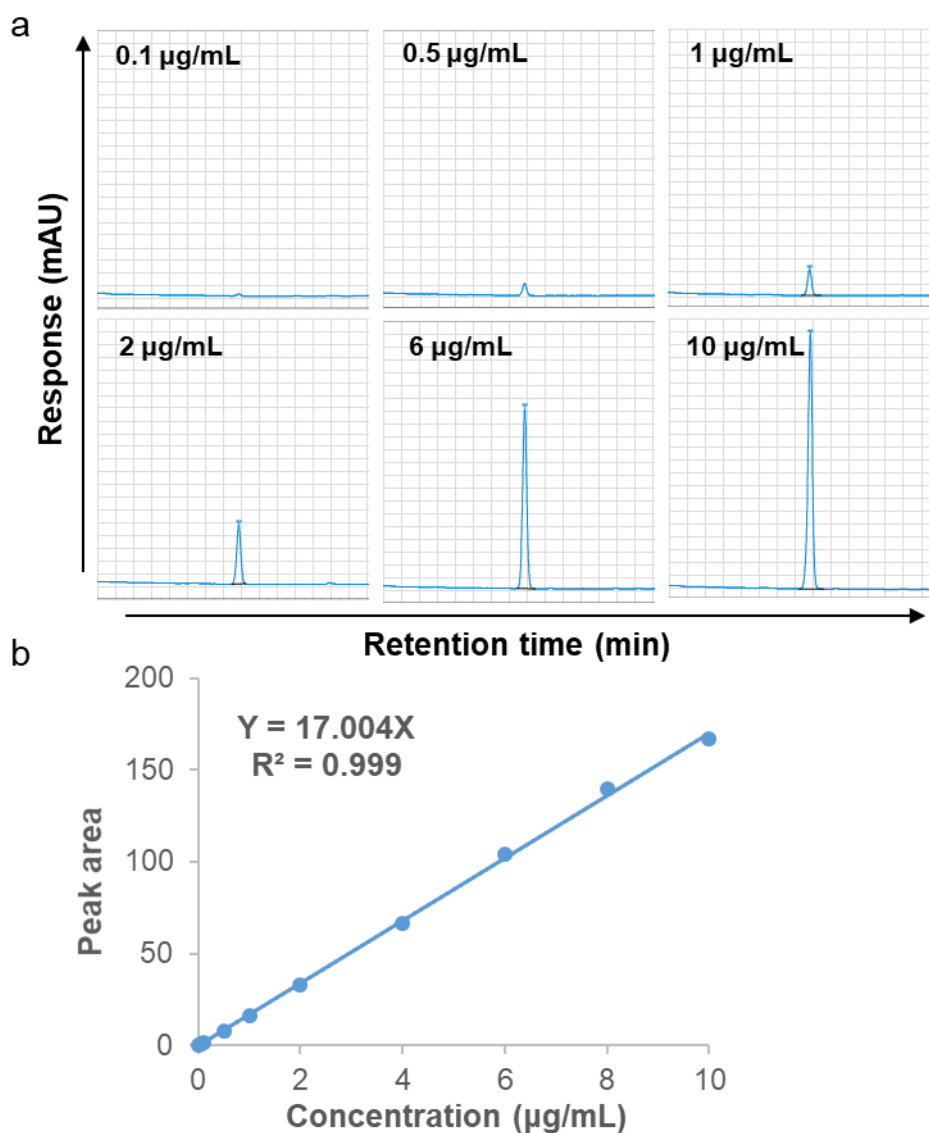
Figure S1. (a) Representative HPLC chromatograms of AZD3965 in water (retention time intervals are from 10.5 to18 min in X axis; response is -0.2 to 20 mAU in Y axis, and peak elution time of AZD3965 appears at 14.5 min). **(b)** Standard curve of AZD3965 peak area over drug concentration. Linearity range: 0.05-10 µg/mL.
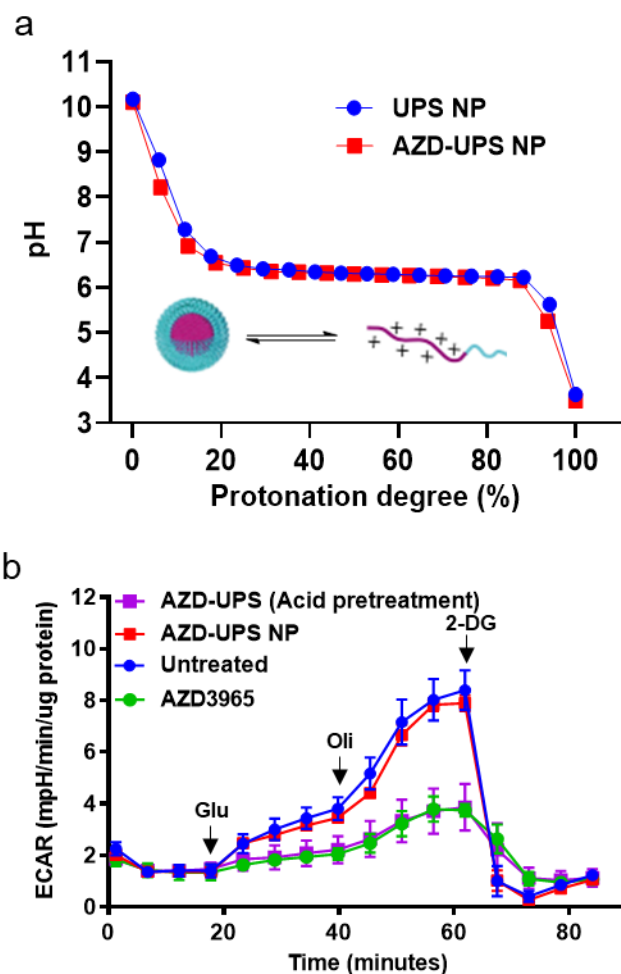
Figure S2. (**a**) pH titration curves of drug-free UPS and AZD-UPS nanoparticles show a sharp pH transition in saline solution. Drug loading did not affect the pH response of the polymer carriers. (**b**) Seahorse analysis of extracellular acidification rate (ECAR) of TC-1 cells with different treatments for 2 h. ECAR data was measured under basal conditions without glucose (Bas), with glucose (Glu), at maximum (Max) with the addition of oligomycin (Oli), and inhibited by 2-DG. Data are presented as means ± SEM, n= 5.
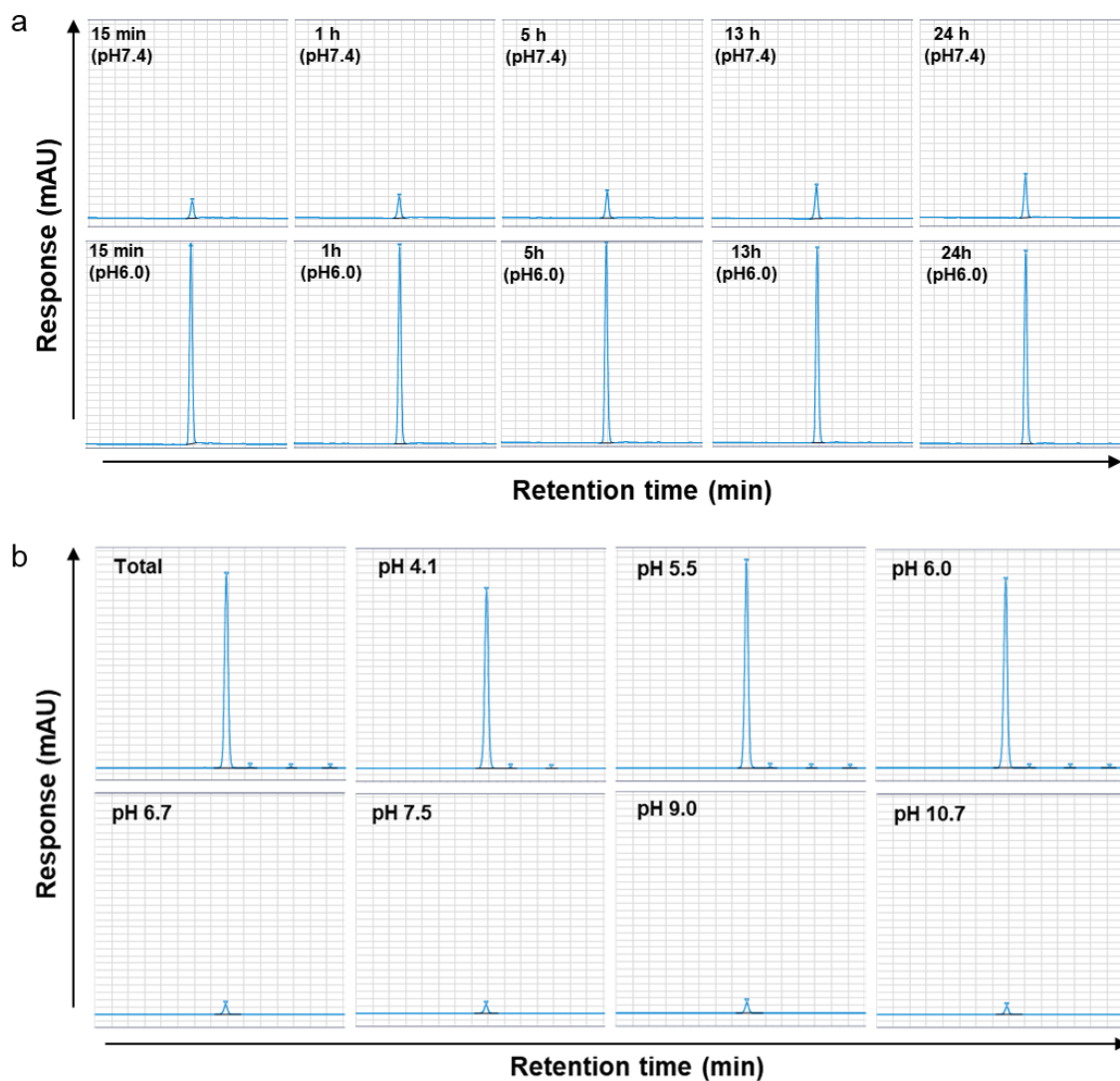
Figure S3. (a) Representative HPLC chromatograms of pH-dependent drug release from AZD-UPS NPs in phosphate buffered saline (PBS) over 24 h at 37 □. At pH 6.0, instantaneous release of AZD3965 drug was observed after micelle dissociation whereas majority of the drug was kept in the micelles at pH 7.4. **(b)** Representative HPLC chromatograms of drug release profiles over different medium pH from AZD-UPS NPs. In a-b, retention time intervals are from 10.5 to 18 min in X axis; response is from -0.2 to 50 mAU in Y axis.
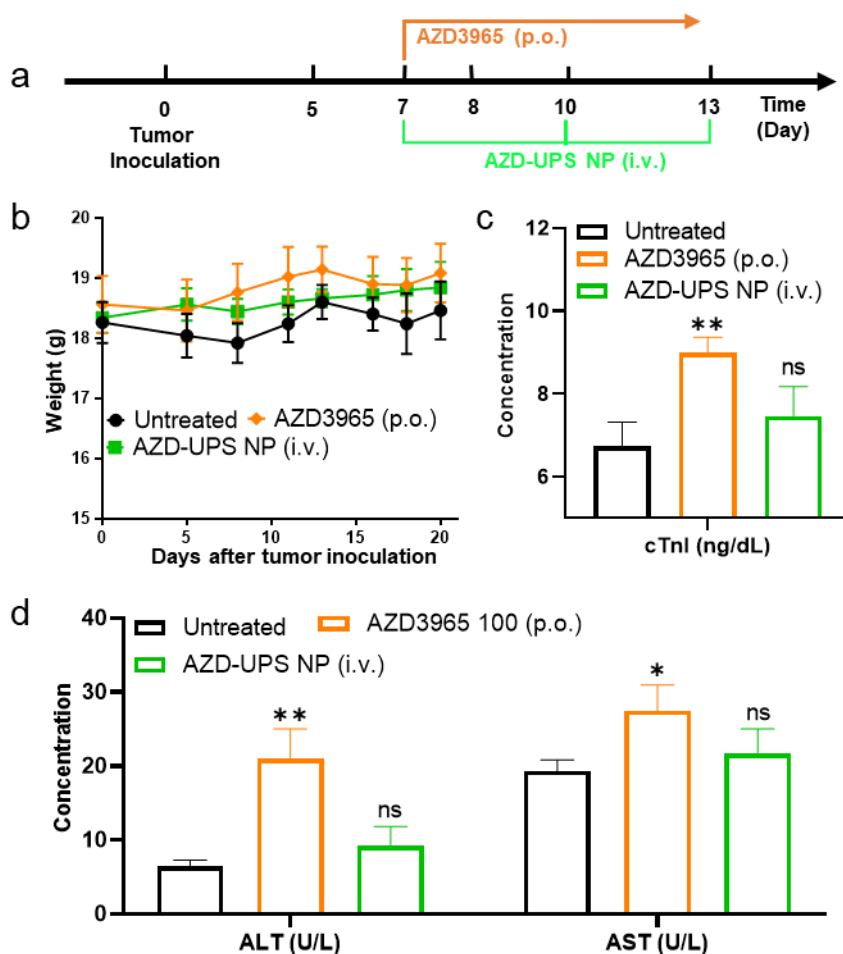
Figure S4. Safety evaluation of oral administration of free AZD3956 and intravenous injection of AZD-UPS nanodrug. (**a**) Scheme of the treatment regimens in the TC-1 tumor model. (**b**) Body weight curve of different group of mice. Cardiac troponin-I（cTnI, **c**）, ALT and AST levels (**d**) in serum of different groups at 4h after last treatment on day 13. Data are presented as means ± SEM, n= 5 in (b). Data are presented as means ± SD, n= 3 in (c) and (d). Statistical significance is calculated vs. untreated group by Student's t-test: **P<0.01, *P< 0.05, ns for not significant.
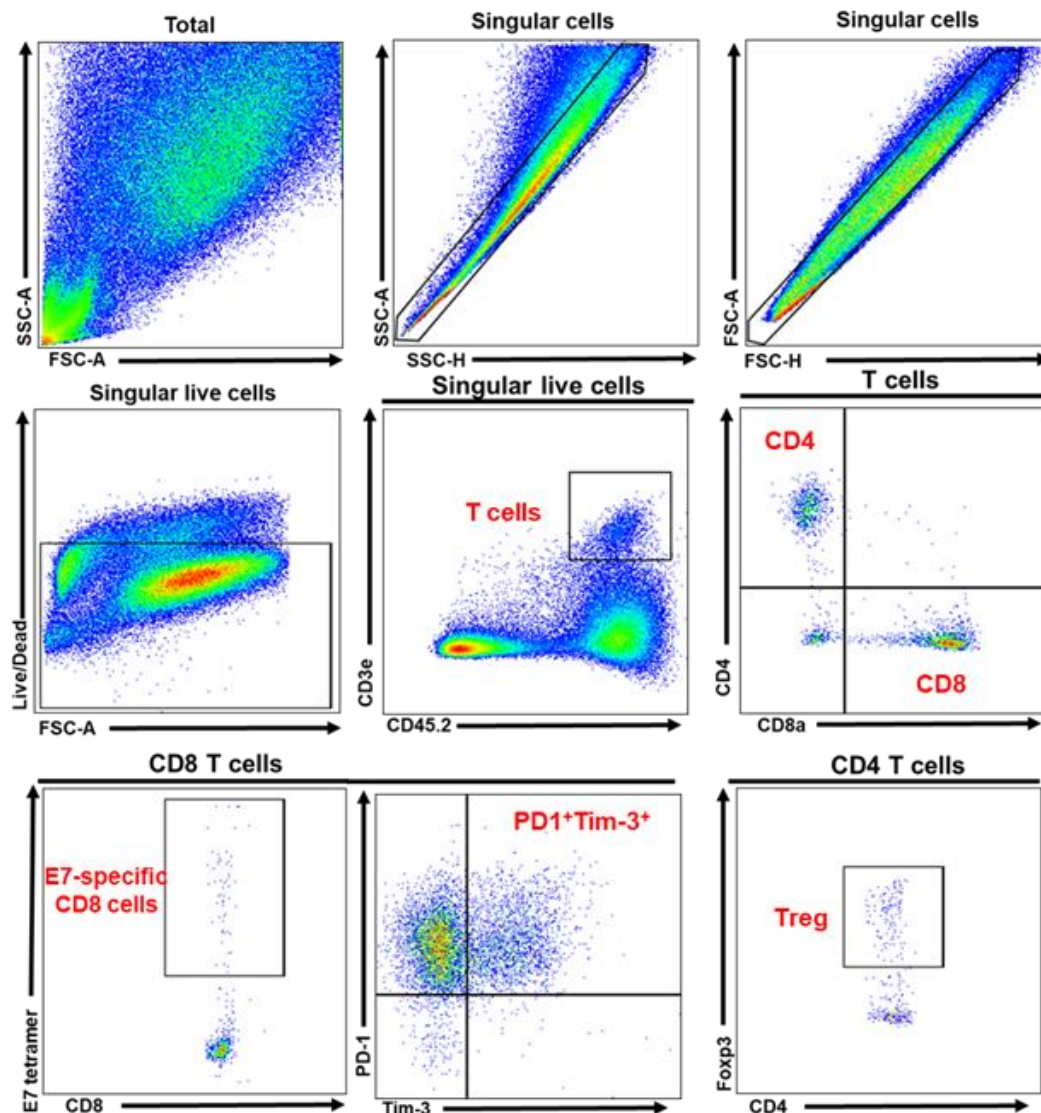
Figure S5. Gating strategy for flow cytometry analysis of living singular cells (live/dead-), T cells (CD45+CD3+), CD4+ T cells (CD4+), CD8+ T cells (CD8+), PD-1^{+}Lag-3^{+} CD8 T cells, PD-1^{+}Tim-3^{+} CD8 T cells, E7-specific CD8+ T cells, Treg(CD4+Foxp3+) in TC-1 tumor from C57BL/6 mice.
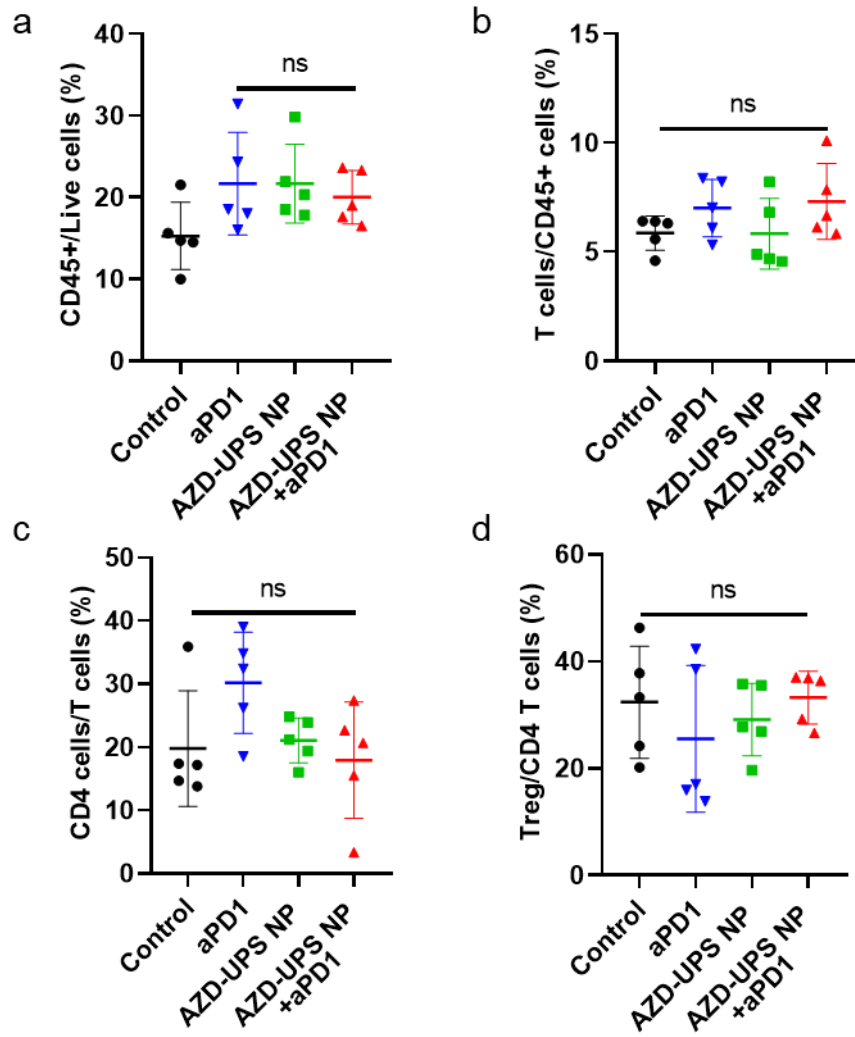
Figure S6. Flow cytometry results show total leucocytes (CD45+) **(a)**, T cells(CD45+CD3+) **(b)**, CD4 T cells **(c)**, and Tregs (CD4+Foxp3+) **(d)** in TC-1 tumors from C57BL/6 mice. Data are presented as means ± SD, n= 5. Statistical significance was calculated by Student's t-test. These cells were found not significant (ns) from any of the treated groups over untreated control.

References

[1]   T. Zhao, G. Huang, Y. Li, S. Yang, S. Ramezani, Z. Lin, Y. Wang, X. Ma, Z. Zeng, M. Luo, E. de Boer, X. J. Xie, J. Thibodeaux, R. A. Brekken, X. Sun, B. D. Sumer, J. Gao, Nat Biomed Eng 2016, 1.

[2]   J. Sun, Y. Xianyu, M. Li, W. Liu, L. Zhang, D. Liu, C. Liu, G. Hu, X. Jiang, Nanoscale 2013, 5, 5262; Q. Feng, J. Sun, X. Jiang, Nanoscale 2016, 8, 12430.